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The Concentration of Diphtheria Toxin by Acid Precipitation

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For the routine preparation of antitoxic sera, the production of an antigen as free as possible from non-specific material is a great advantage. Immunologists are agreed that the extraneous substances in a low grade toxin solution hinder the production of immunity (*M.R.C. Report*, 1923). Hitherto very few efforts have apparently been made to attempt the preparation of such antigens on the large scale, chiefly because the various precipitating agents employed either only partially precipitated the toxin or else, *e.g.* alcohol (Banzhaf, 1920), were too expensive to permit of their use. Glenny and Walpole (1915) described a method for converting low grade filtrates to those of high titre by means of precipitation of the filtrates with dilute acetic acid after pressure dialysis in a collodion membrane, the precipitate being dissolved in dilute caustic soda. As shown by the present authors (Watson and Wallace, 1924) this preliminary dialysis, which is a tedious process, may be dispensed with if the amount of acetic acid added is considerably increased. It was shown that for the special medium employed, *i.e.* Hartley's modification of the Douglas medium (Hartley, 1922), the amount of nitrogenous material precipitated by glacial acetic acid from the filtrates gradually increases with growth until a maximum is attained. Further, it was shown that by carefully defining the conditions of the precipitation and separation it was possible to recover most of the toxin from the filtrates by this means. The present paper describes a method, founded on the experiments described previously by Watson and Wallace (1924), that has been used on a large scale and over a long period for the preparation of a powerfully toxic material considerably more free from non-specific material than the original filtrates. Most of the experiments were carried out before the general recognition of the work of Ramon (1922, 1923, etc.), who has described a method for the titration *in vitro* of antitoxic sera against toxic filtrates of *B. diphtheriæ*. It seems possible that this technique will ultimately prove of more value to the immunologist for the rapid and accurate determination of the real combining value of sera and toxic filtrates than any of the animal tests on which he has, up to the present, been compelled to rely. The estimation of the Ramon units in the toxic filtrates and reconstructed antigens would probably have been of more value than the number of "Lr/500 doses per c.c." which for want of a better technique has been adopted almost exclusively in the present work.

Method employed.

The method consists in the separation and collection of the nitrogenous material precipitated by glacial acetic acid from the well-cooled filtrates of the bacillus. For the collection of the precipitate

the ordinary hand or motor driven Sharples centrifuge has been employed and has proved very satisfactory.

Formation of precipitate.—It has been shown in a former communication (1924) that during the growth of P.W. 8 strain of *B. diphtheriæ*, in addition to hydrogen ion concentration changes, several interesting changes in nitrogen metabolism take place in the culture medium. Of these the most important are the increase in the amount of nitrogenous material precipitable by glacial acetic acid, and the amount of amino-nitrogen as determined by the Van Slyke method.

TABLE I.

No.	Type of medium.	Milligrams Van Slyke N. per 10 c.c.		Milligrams Precipitable N. per 300 c.c.		Toxicity.	Strain.
		Before growth.	After growth.	Before growth.	After growth.	Lr/500 doses per c.c.	
1766	Tryptic digest of horse muscle.	9·62	16·62	8·71	12·2	2500	...
1767	,, ,,	9·60	15·67	10·95	12·12	2000	...
1771	,, ,,	12·59	18·06	3·25	17·11	4000	79
1771	,, ,,	12·59	20·34	3·25	29·04	5000	103
1771	,, ,,	12·59	17·26	3·25	23·88	3333	106
1769	Witte peptone broth .	5·54	9·25	13·33	...	5000	...
1773	,, ,,	5·07	6·13	12·89	17·08	200	79
1777	,, ,,	5·17	6·49	13·1	12·4	1250	103
1775	,, ,,	4·05	7·80	12·24	16·10	less than 100	106
1780	,, ,,	5·33	5·49	17·28	...	100*	...
1783	Abt and Loiseau broth .	5·23	7·69	4·76	7·72	1666	79
1786	Difco peptone broth .	4·98	5·94	5·52	13·36	less than 100	106
1787	Parke-Davis peptone broth.	7·40	8·7	...	6·5	...	106

* This filtrate on concentration by the method outlined to one-twentieth of the original volume gave a solution of very high Ramon value suggesting that the original filtrate might have been of high combining value but of low toxicity.

Table I. shows the analysis of various culture media made by different methods, and brings into prominence these typical changes which are found during the growth of the powerfully toxin-producing strain of *B. diphtheriæ*. It will be seen that parallel with the production of amino-nitrogen as growth progresses we get an increasing amount of nitrogen which is precipitable by glacial acetic acid. It must be emphasised at this point that the production of this "precipitable nitrogen" is a function of the growth and the particular strain of the bacillus rather than of toxin production. When the various factors which influence toxin production are working smoothly, we find that this precipitable nitrogen contains most of the toxin, if precautions are taken regarding its separation and recovery. It will be seen also from the above table that toxins made from "digest" media (Watson and Wallace, 1923) contain a comparatively large

amount of precipitable nitrogen. These are more easily dealt with, and most of the work of the present paper was carried out with this type of filtrate. Other types of filtrates which have been used have given equally good results, although when the precipitate is small the mechanical difficulty of completely recovering the precipitate from the Sharples machine makes the loss of precipitate more appreciable than when large amounts of precipitate are present.

The filtrates for concentration are placed in large double Winchester quart bottles, four litres per bottle, and the contents cooled to just above freezing point. The required amount of glacial acetic acid is then added, and the bottles are allowed to stand in the cold room. After a short time varying from five minutes to two hours a precipitate commences to flocculate and then settles to the bottom of the bottle. Occasionally the precipitate settles out in well-defined strata in a form closely resembling that associated with the well-known Liesegang phenomenon.

Separation of precipitate.—With as much expedition as possible the contents of the bottles are then run through the centrifuge. The time of spinning depends on the type of feed nozzle employed. For complete separation it is advisable to use the smallest nozzle, although for routine separation, if a high speed can be maintained with the machine, an equally efficient separation can be obtained more rapidly with a medium-sized nozzle. When the separation is complete the machine is slowed down and the precipitate is removed. It is preferable to carry out all these operations at a temperature just above freezing point. In practice, however, this is a matter of some difficulty and the recovery is nearly as complete if the centrifugation of the cooled precipitated filtrates is carried out as quickly as possible at room temperature, and ice-cooled caustic soda used for the solution of the precipitate.

Removal and solution of the precipitate.—The precipitate is scraped from the sides of the cylinder into glass mortars containing a small amount of ice-cooled water. It is then thoroughly emulsified with the water and previously cooled dilute caustic soda added until solution just takes place. Great care is taken to keep the P_H of the solution below 8·4. This may be done by checking from time to time with a suitable indicator, *e.g.* cresol red. It is usually possible to dissolve the precipitate completely so that the final solution is perfectly clear. The P_H is adjusted to 8·4, 0·5 per cent. phenol and 0·85 per cent. salt added, and this solution constitutes the concentrated antigen. The volume of the solution is adjusted according to the strength of toxin required. For a given type of filtrate, after a few experiments, the toxicity of the final product may be predicted from the toxicity of the original filtrates.

The following experiments describe certain aspects of the process and show the effect of varying the factors involved.

1. The effect of varying the amount of glacial acetic acid added to the filtrates on the amount of material and "toxin" precipitated.

Table II. shows the results of varying the amount of acid added on the ultimate yields of toxin.

TABLE II.

	Percentage of glacial acetic acid added.	Toxicity. Lr/500 doses per c.c. Original filtrate.	Toxicity. Lr/500 doses per c.c. Final solution.	Precipitable nitrogen in 300 c.c. filtrate.
A.	1	667	4000	15·54 mgrms.
B.	3	,,	4000	20·3 ,,
C.	5	,,	3333	18·76 ,,
D.	10	,,	3333	17·22 ,,
F.	15	,,	2500	16·52 ,,
H.	20	,,	2200	14·84 ,,
K.	30	,,	...	Negligible.

For this experiment six four-litre volumes of filtrates containing 667 Lr/500 doses per cubic centimetre were precipitated with varying amounts of glacial acetic acid at 5° C. The precipitate flocculated and settled in half an hour. It was then separated by centrifugation as described above, and dissolved in ice-cold caustic soda to one-tenth the original volume. The number of Lr/500 doses per cubic centimetre of this solution was then determined.

It will be seen that the best recovery of toxin for this type of filtrate is effected by treatment of the filtrates with from 1 to 3 per cent. of glacial acetic acid. This result is typical of many others carried out on similar lines and for filtrates prepared from media made by the tryptic digest of horse muscle the best results are normally obtained with 3 per cent. glacial acetic acid. For most types of filtrates, however, which have been made from media which have supported good growth, a preliminary experiment of this type is necessary before the amount of acid necessary for the maximum recovery of toxin can be decided upon.

The last column represents the amount of nitrogen precipitable from 300 c.c. of the original filtrates under the following conditions. This amount of filtrate was placed in each of seven flasks and the quantities of glacial acetic acid added. The precipitated material was rendered more easily filterable by placing the flasks in water at 100° C. for ten minutes. The flasks were then removed and cooled to room temperature, after which they were filtered. The nitrogenous material was washed with 300 c.c. of distilled water after which the washings contained no detectable traces of nitrogen. The precipitate was washed into a Kjeldahl flask and a nitrogen estimation carried out in the usual way. Three per cent. of acid precipitates most nitrogen, and as the amount of acid is gradually increased the amount of precipitate becomes less until with 30 per cent. acid the precipitate is negligible.

2. The effect of varying the temperature of precipitation, etc. on the yield of toxin from filtrates of the bacillus.

It has been stated above that ice-cold conditions during the precipitation and solution of the toxic material considerably improve the yields of toxin. Table III. shows the results of one experiment which demonstrates this fact. For this experiment the precipitations were carried out at the different temperatures, the centrifugation by the Sharples machine at room temperature and the re-solution of the precipitated material at the three temperatures stated.

TABLE III.

	Temperature of precipitation.	Toxicity. Lr/500 doses per c.c.		Volume concentration.
		Original filtrate.	Final solution.	
A.	2° C.	1429	16,666	20
B.	16° C.	1429	8,333	20
C.	37° C.	800*	2,857	20

* This filtrate lost nearly half its toxin during the incubation at 37° C. for twenty-four hours.

It will be seen that the lower the temperature the more complete is the recovery of toxin. This confirms the previous results (Watson and Wallace, 1924), although the recoveries are not so high owing to the fact that under the large scale conditions of routine concentration it is impossible to maintain the low temperature conditions so necessary for the maximum recoveries of toxin.

For the experiment twenty-one litres of filtrates of the bacillus (made from medium prepared by a tryptic digest of bullocks' hearts and containing 1429 Lr/500 doses per cubic centimetre) were selected. This was distributed into six bottles, three and a half litres per bottle. Two bottles were cooled to 2° C. and precipitated in the cold room with 3 per cent. glacial acetic acid. The precipitate was allowed to coagulate, and after standing thirty minutes under cold room conditions was separated by centrifugation in the Sharples machine as rapidly as possible. The precipitate was then dissolved in previously cooled dilute caustic soda to a P_H of 8·4. A further seven litres of filtrates were precipitated under room temperature conditions, the precipitate being dissolved in the same volume of caustic soda to a P_H of 8·4. Finally, a third quantity of filtrate was precipitated at a temperature of 37° C. and precipitation and re-solution carried out at this temperature.

It will be seen that the most toxic product was obtained when the various operations were carried out as nearly as possible under cold room conditions. Precipitation at room temperature or higher temperatures usually does not give such efficient recoveries of toxin. Occasionally, with certain types of toxin it is found that temperature plays a very

minor part in the recoveries effected. As a general rule, however, more consistent and satisfactory recoveries are effected by keeping the temperature as low as possible.

3. The effect on the yields of toxin of standing for varying periods of time before separation of precipitate.

When 3 per cent. glacial acetic acid is added to toxic filtrates of *B. diphtheriæ* (P_H 7·8-8·4) a cloudiness is produced but very rarely a precipitate at once. After a few minutes, however, a precipitate begins to flocculate, the time of appearance depending on the type of medium used, temperature of precipitation, etc. Where the medium employed has been prepared by tryptic digestion of horse muscle (or other medium which has supported good growth) the precipitate is heavy and settles out in a short time. After standing for some time the precipitate becomes granular and after several days the supernatant liquid is perfectly clear, under which conditions the precipitate may be readily recovered by siphoning off this supernatant liquid. Under these conditions, however, a proportion, but not all, of the toxin is lost as will be seen from table IV.

Table IV.

	Period of standing before centrifugation.	Toxicity. Lr/500 doses per c.c.		Volume concentration.
		Original filtrate.	Concentrated solution.	
A.	0 days.	1429	16,666	20
B.	2 ,,	1429	7,111	20
C.	4 ,,	1429	10,000	20
D.	6 ,,	1429	11,111	20
F.	9 ,,	1429	6,250	20
H.	3 months.	1429	7,111	20

For the experiment, four litres of the filtrate were precipitated with 3 per cent. glacial acetic acid and left at cold room temperature for varying time periods, after which they were spun down in the machine and dissolved under similar conditions. The number of Lr/500 doses per cubic centimetre of the reconstructed antigen was then determined.

The effect of leaving the precipitate in the acid medium is reflected in the toxicity of the precipitated material. Though some loss of toxicity has occurred, it is much less than the general statements current as to the extreme instability of diphtheria toxin in the presence of acid would lead one to expect.

The nitrogen content of the solutions remained practically constant, showing that on prolonged standing no further precipitate is produced.

4. The nitrogen content of the precipitated material.

It is reasonable to assume that the freer the filtrates are from extraneous substances, the greater is the value of the antigen from the point of view of the immunologist. As deleterious substances are probably nitrogenous, this freedom from extraneous substances has been measured as strength of toxin per milligram of nitrogen. One of the chief features of the above process is the very low nitrogen content of the reconstructed antigens.

TABLE V.

Number.	Milligrams nitrogen per c.c.		Lr/500 doses per c.c.		Concentration of toxin per mgrm. nitrogen.	Volume concentration.
	Filtrates.	Concentrated solution.	Filtrates.	Concentrated solution.		
BJ 1760	5·236	1·284	1111	10,000	37	20
BJ 1764	4·270	1·557	1000	10,000	27	30
BJ 3507	4·690	0·636	1429	16,666	86	20
BJ 3526	4·480	0·776	1667	11,111	38	20
BJ 3531	3·860	3·996	1667	67,000*	39	80

* The potency of this toxin was such that the intracutaneous injection of one five-millionth of a cubic centimetre in the shaven flank of a guinea-pig caused a typical skin reaction.

Table V. shows the nitrogen figures for a few preparations of this material. It will be seen that where the original filtrates have been concentrated twenty times by volume, the nitrogen content has been reduced to approximately 15 to 20 per cent. of the original filtrates. This corresponds to a concentration of toxin per milligram of nitrogen of from thirty to eighty. For a long series of these reconstructed antigens the average concentration has been forty. The final product is considerably freer from non-specific material than the original filtrates, and prima facie, therefore, should be a much more satisfactory antigen. It has been shown in a previous paper (Watson and Wallace 1924) that only by keeping the temperature consistently low throughout the operations can high yields of toxin as determined by Lr/500 values be expected. Under the routine conditions of concentration this is very difficult, with the result that the recoveries given in the table are not so high as they would be if the various operations had been carried out at consistently low temperatures. It is possible that the change from the toxic to some "toxoid" state which takes place slowly in ordinary filtrates is considerably accelerated in these reconstructed antigens. The Lr/500 values become misleading if this change has proceeded far. It would seem that the process described, although it involves a certain loss of toxin during the operations, results in the production of a product in which the concentration of "toxin" per milligram of nitrogen is very high. This comparative freedom from

extraneous material should make these solutions of value for the study of the chemical nature and reactions of the exotoxin.

5. The reaction between the reconstructed antigens and antitoxic sera.

The discovery by Ramon (1922, 1923, etc.) of a method of titrating antitoxic sera against toxic filtrates of *B. diphtheriæ in vitro* has been investigated by several workers. S. Schmidt (1923) confirmed Ramon's results and showed that in the titration of a group of antitoxic sera close agreement was obtained between the values obtained by the Ramon method and those by animal tests. Glenny and Okell (1924) suggest that the amount of toxin which is exactly neutralised by one unit of antitoxin when determined by the method of Ramon should be termed the Lf value of that toxin. These workers showed that for the same serum the ratio of the Lf value of a toxin to the Lo or Lr values was constant for the majority of toxins examined by them but they pointed out that with other sera and with certain samples of old toxins, the ratio is not the same. The group of toxins in which the ratio was found to hold had been prepared by similar methods, and they suggest that different methods of preparation of the toxins may account for the variations in the ratio.

When we compare the two values for a series of the reconstructed antigens it is found that no constant ratio exists between the two even though the solutions were in the first place made by analogous methods. It has been shown that, at the expense of a certain amount of toxin, highly toxic antigens may be obtained by the re-solution of the precipitate obtained by the action of glacial acetic acid on the well-cooled filtrates of the bacillus. The Lf values of these solutions are consistently high, but the actual toxicity as determined by M.L.D., or apparent strength as determined by Lr/500 methods, is dependent on the temperature, speed, etc., at which the concentration has been carried out.

Table VI.

Serial number.	Lf units per c.c.	Toxicity. Lr/500 doses per c.c.	Ratio. Lr/500 to Lf units per c.c.
WSC. 7 (2)H	59·0	7,111	121·0
CJ. 1	20·0	10,000	500·0
CJ. 9	17·7	3,333	188·0
CJ. 10	15·0	4,000	266·0
CJ. 12D	42·0	11,111	265·0
CJ. 16C	56·0	10,000	178·0
CJ. 17	170·0	4,000	23·5
CJ. 18	130·0	3,333	25·6

Table VI. shows the two values for a group of reconstructed antigens and it will be seen that no common relationship exists between them.

It would appear that outside conditions of temperature, etc., have resulted in the change of the active principle from the toxic to the toxoid state. The latter change has possibly not affected the combining value of the filtrate, as is suggested by the Lf values. It would seem that the animal tests show that the toxin of the filtrates is very susceptible to changes of temperature, etc. These changes, whilst affecting the toxicity, because they do not affect combining power, may not interfere with the antigenic value of the solution. It has been the experience during the present work to find the toxicity of the various reconstructed antigens rapidly falling off with increases in temperature and time (Watson and Wallace, 1924), whereas the Ramon flocculation titrations show a tendency to remain steady provided the temperatures to which the solutions have been exposed have not been too severe. It may be that these solutions which before the recognition of the Ramon test would have been regarded as low grade antigens may be found on experiment to be quite useful.

6. The concentration of chemically modified toxins.

That toxic filtrates of *B. diphtheriæ* may be so modified that whilst the actual toxicity is very considerably reduced the antigenic properties remain unimpaired has long been recognised. Glenny and Sudmersen (1921) by modification with formalin were able to inject 5 c.c. of a filtrate into a guinea-pig without producing any injury although previous to the treatment the filtrate had killed in dilutions of $\frac{1}{200}$ of a cubic centimetre.

When the concentration process outlined above is applied to filtrates modified with formalin it is found that provided the precipitate has not been left for too long a period before centrifugation, a perfectly clear reconstructed antigen is obtainable and that the Ramon units have also been concentrated. Most of the work of the present paper was carried out before the general recognition of the value of Ramon's work. Possibly a proportion of the loss occasioned during the process is due to the change of the active principle from a toxic to a toxoid state. If this change has proceeded sufficiently far the Lr/500 values would appear to indicate a loss of the active principle, and it would probably have been more satisfactory to employ the Ramon values as criteria of the activity of the various filtrates and reconstructed antigens. It is possible by precipitation methods to obtain solutions which give very high Lf values. Thus by using the method described above, reconstructed antigens which gave Lf values in dilution as low as 0·017, 0·005 and even 0·002 c.c. have been obtained. Work is being continued on these solutions, which must, if the Ramon titrations are proved to be criteria of antigenic value, be of great importance to the immunologist.

Summary.

1. A rapid method for the concentration of the toxic material free from the greater proportion of the non-specific constituents of the filtrates of *B. diphtheriæ* is outlined. A considerable concentration of toxin per milligram of nitrogen is effected by the process.

2. Certain aspects of the process showing the effect of varying the factors involved are described. A marked feature of the method is the part played by the temperature at which the various operations are carried out. Greater yields of toxin as determined by Lr/500 values are usually obtained if the temperature is kept near freezing point throughout the process.

3. A very useful filtrate for this type of work is that prepared from medium produced by the tryptic digestion of horse muscle. Most filtrates, however, would appear to yield good results with the method.

4. Solutions which are practically non-toxic but which have very high values as determined by the Ramon flocculation method are obtained when filtrates which have been modified by formalin are used for concentration.

5. In addition to their value as antigens, their freedom from extraneous material should make these concentrated solutions of value for the study of the nature and reactions of the specific toxin of the bacillus.

We are indebted to our colleague Mr Glenny for the work done on the determination of the Ramon values

REFERENCES.

Banzhaf	Pathogenic Micro-Organisms. Park and Williams, 1920, p. 175.
Glenny and Okell	This *Journal*, 1924, vol. xxvii. p. 187.
Glenny and Sudmersen . .	*Journ. of Hygiene*, 1921, vol. xx. p. 175.
Glenny and Walpole . . .	*Biochem. Journ.*, 1915, vol. ix. p. 298.
Hartley	This *Journal*, 1922, vol. xxv. p. 479.
Medical Research Council .	Diphtheria, 1923, p. 113.
Ramon	*Compt. rend. Soc. Biol.*, 1922, vol. lxxxvi. pp. 661, 711, and 813.
,, ...	*Compt. rend. Soc. Biol.*, 1923, vol. lxxxviii. p. 167.
,, ...	*Compt. rend.*, 1923, vol. clxxvi. p. 267.
,, ...	*Compt. rend. Soc. Biol.*, 1923, vol. lxxxix. p. 2.
Schmidt	*Compt. rend. Soc. Biol.*, 1923, vol. lxxxviii. p. 105.
Watson and Wallace . . .	This *Journal*, 1923, vol. xxvi. p. 447.
Watson and Wallace . . .	This *Journal*, 1924, vol. xxvii. p. 276.

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